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Xenon as a neuroprotectant in traumatic brain injury

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**14. ABSTRACT**
The purpose of this novel study is to determine if xenon has a neuroprotectant effect in an vivo animal models of TBI. The project scope is an early proof of concept in rat models of xenon neuroprotection. The Specific Aims are to determine the effect of inhaled xenon on brain histopathology, behavior, in short- and long-term fluid percussion (FP) and controlled cortical impact (CCI) rat models of TBI compared to controls. The scope of this project is proof of concept in a rat model. Major findings and progress: IACUC and VA Research Approvals obtained. We developed a recirculation xenon device for this project. Xenon and devices for behavioral studies acquired. Initial experiments using the CCI model do not show significant improvement, rather the contrary, in histopathologic or behavioral outcomes between controls and xenon-treated rats. We are proceeding with the FP model to assess these histopathologic and behavioral outcomes in the short term, and, if promising the long-term.

**15. SUBJECT TERMS**
Traumatic brain injury xenon neuroprotection animal model

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**INTRODUCTION:** Xenon has neuroprotective effects, through blocking multiple neuronal receptors (NMDA, AMPA, kainite and others) to block excitotoxicity, but is also anti-apoptogenic, may regulate cerebral blood flow, blocks excitotoxic dopamine release, and has anti-inflammatory effects, as well as other mechanisms. The progressive secondary neuronal damage from TBI is dependent upon these mechanisms antagonized by xenon. The purpose of this novel study is to determine, at the level of proof of principle, if xenon has a neuroprotectant effect in *in vivo* animal models of TBI.

**BODY:**

**OBJECTIVES/SPECIFIC AIMS.** We will test the hypothesis that inhaled xenon administered after TBI reduces neurologic and behavioral deficits in two *in vivo* rat models.

**Task 1. Determine the effect of inhaled xenon on brain histopathology in short-term fluid percussion (FP) and controlled cortical impact (CCI) rat models of TBI compared to controls.**

**1a. Approvals will be obtained from IACUC (BWH) and Research and Development Committee (VAMC).**

We have obtained these approvals.

**1b. Equipment and xenon procurement.** Manufacture and procurement of the devices and xenon for this project required a longer time than anticipated, due to the specialized nature of all the equipment and gas required. The Beam Walk Device and Dragonfly (model HPD-1700) Variable Pressure Waveform Generator with transducer, charge amplifier, and remote triggering device were manufactured by Dragonfly and were delivered. A xenon/oxygen analyzer was acquired from Alpha-Impex, Oy, Finland, but required a power inverter, battery and charger, which were also obtained. Industrial grade xenon with regulator and flow meter were acquired from Air Liquide, by special arrangement. Administration of the xenon required design and manufacture of a specialized xenon recirculation device. This unique design was developed by Dr. Jose Venegas at Massachusetts General Hospital (MGH) and the Bioengineering Workshop at MGH constructed the device.

**1c. Methods development.** We have developed a unique method of xenon gas administration to a single rat at a time. We have consulted with researchers at the Imperial College of London to review their method of administration of xenon, but developed our own method. The device is a box, with a small fan, trays of silica for water vapor removal, and soda lime for CO2 absorption, input for oxygen and xenon. A balloon is inside the device which contains air and removal of a volume of gas (either oxygen or xenon) will allow input of an equal volume of gas into the recirculation device. The box is flushed with 100% oxygen and the concentration of xenon can be controlled by removing a known volume from the balloon inside the box. Since the volume of the box is also known, the concentration of the xenon added to the box will be proportional to the volume removed from the balloon. This is verified by direct measurement of oxygen and xenon using the Alpha-Impex xenon/oxygen analyzer. Decrease in oxygen concentration can be adjusted by adding oxygen to the system. The oxygen analyzer underestimates by about 3% and the xenon analyzer underestimates by about 4-5%, despite calibration. This xenon/oxygen analyzer is currently recognized as the most accurate on the market, in my discussions with colleagues familiar with xenon administration and analyzers. We are proceeding with 3 hours exposure of xenon at 50%/oxygen at 50% vs. room air.

**1d. Conduct of CCI trials.** Study planned 14 rats in each TBI arms (moderate CCI, xenon, air) and 14 in each sham injury arms (xenon, air). Each surgery day has at least one animal from each group. Animals are sacrificed in < 1week using brain perfusion /fixation method. The method follows:

**C*ontrolled Cortical Impact Summary**:* Rats are subjected to TBI utilizing the controlled cortical impact model (CCI). This model utilizes a non-penetrating, localized deformation of the cortex induced by a pneumatic impactor. Animals subjected to injury with the CCI model receive a unilateral cortical impact

to the left parietal cortex utilizing a beveled impacting tip (5 mm diameter) with a contact velocity of 3.5 m/sec and a depth of ~2 mm (final depth to be set pending ongoing experiments). This diameter tip and velocity is set to produce a consistent and pronounced morphological alteration in the cortex stopping just short of the hippocampus (ie, a moderate injury). Sham animals will receive a craniotomy exposing the cortical surface identical to the impact group. The impactor tip is lowered manually to the cortical surface but no impact will ensue.

We have completed all set-up and necessary injury titration, as well as procured and tested the needed devices (beam, fluid percussion device, xenon chamber). As has been discussed, these delayed start of Xenon experiments. We have run an N of 4 for all groups in the controlled cortical impact (CCI) model. Briefly, there are two key results: first, that there is substantial CCI-induced hippocampal injury and second, is that xenon in this model with this degree of injury is generally worse than or at best equal to air, and this appears very unlikely to change in follow-up experiments. Thus, using the adaptive design as intended, we consider three possible courses of action: (i) Continue short (1 week) study with xenon; (ii); Switch to long CCI study, or; (iii) switch to fluid percussion (FP) short term study.

Option (i): As noted above, the short study seems very unlikely to yield useful data, and even the most protective phenotype that we see seems unlikely to reach significance in the context of a multiple comparison correction. While we can return to this option later, it currently seems less appropriate.

Option (ii): If we run the FP short study and it is positive, we would clearly want to follow-up with the FP long study. If it is negative, or if it is neutral as is the CCI study, we can reconsider either model (or neither) at that time. We note, however, that the substantial CCI-induced hippocampal injury appears unaffected at best in Xenon-treated animals, suggesting that Xenon is unlikely to show substantial protection in long-term studies in this model. Thus, in neither case does running the CCI long-term study seem financially, practically, or ethically prudent. We therefore plan to, at least temporarily, drop all CCI work, pending the final cylinder work.

Option (iii): Thus, proceeding with short-term FP appears to best fit the objectives of out project. We are currently titrating this model with the newer device.

Additional options: We will consider weight-drop and combination projects, and altered Xenon dosing. Any changes from the original SOW will require approval by the granting agency.

*Supporting Notes and data*: Basic animal recovery from surgery/TBI: There is no obvious pattern in diet or weight gain/loss.

*Blinding and allocation strategy:* Experimental conditions (e.g., impact depth, gas etc) for any given day are chosen at random (random number chart). We cannot blind our surgeon to impactor depth and gas delivery as only one person is in the room. A blinded examiner will do all slide analysis. We follow an "intent-to-treat" style analysis, although we will consider retaining a condition longer if the initial poor results appear largely due to deaths prior to treatment (unlikely in this paradigm).

1) *Adaptive design.* We run all analyses with a broad adaptive design, meaning that we stop if it becomes statistically apparent that xenon is either protective or not. This is standard in Dr. Kristal's IACUC approved TBI protocol. More specifically, we run all studies with a continual adaptive design. That is, we seek to stop studies as soon as possible (the equivalent of futility trials in humans), so as to save critical resources, e.g. money, time, effort, and to enable deeper studies of promising conditions or reagents and further reduce animal use. From the time we run the 3rd replicate of each condition, we begin to actively monitor the study to determine if we can stop testing some conditions. By the time we reach an N of 4, we can use Bayesian and frequentist adaptive designs, the latter using the program EAST from Cytel. We will continue to monitor after each set of animals has been run. This approach allows us to maximally reduce animal usage, and efficiently proceed to the next model or condition.

**1g. Conduct of FP trials.** We are proceeding with studies utilizing this model. However, there are no data available at this point in time, but will in the near future. Study involves 14 rats in each of the TBI arms (moderate FP, xenon, air) and 14 in each sham injury arms (xenon, air). Each surgery day has at least one animal from each group. Animals will be sacrificed in < 1week using brain perfusion /fixation method. The method for fluid percussion follows: Rats are subjected to FP-induced TBI. Rats are subjected to TBI utilizing the lateral fluid percussion (FP) model. Briefly, the skull is opened via a craniotomy (4.8 mm) centered between the bregma and lambda and between the sagittal suture and right temporal ridge and fitted with a Leur-loc fitting and the animal is attached to the Dragonfly (model HPD-1700) Variable Pressure Waveform Generator and subjected to an injury at ~2.5 atm. (Note: final pressure to be set pending final methods development). Sham treated animals are treated identically except for the pressure wave. Blinding and allocation strategy, andadaptive design as in section 1d.

**1e. Histopathologic assessment** Dissected brains are kept in Bouin's fixative before embedding. Blocks are sectioned and stained with hematoxylin and eosin (H&E). We examine and score injured and non-injured cortex/white matter and hippocampus. Damaged volume is assessed both qualitatively (hippocampus is/is not damaged) and quantitatively (area of region of injury. We note that our histopathology is currently done using only H&E staining, but we will be testing a secondary series of stains (eg., amino-cupric silver, flourojade, GFAP). In general, samples are fixed immediately and go to the pathology lab approximately 1 week after sacrifice. They are ready for microscopic study about 2 weeks later.

***Results with hemotoxylin and eosin staining:*** All shams appear perfect, with no obvious effects on the brain. All TBI animals have at least partially cavitating lesions in the cortex with substantial dead/dying cells in the thalamus and CA3 region of the hippocampus. Thus injury level was successfully attained. One air treated animal was considered by the (blinded) pathologist to have a milder injury than the other 7 animals, and 3 of the 4 xenon treated animals were described as having more severe injury than their air-treated counter parts.

**Task 2. Determine the effect of inhaled xenon on behavior in short-term fluid percussion (FP) and controlled cortical impact (CCI) rat models of TBI compared to controls.**

**2a. Conduct of trial** as in Task 1. *Data from FP are not available yet for the outcomes below.*

**2b: Neuroscore/SNAP assessment with CCI:** This is a short term assessment of neurological function. Neuroscore/SNAP is a standard assessment for all TBI studies, and it will be conducted on days 1 and 3 post injury. Neuroscore as described by Hoover *et al* is a composite score based on combining scores from tests of forelimb reflex, hind limb flexion, lateral pulsion, and ability to stay on an angled board. We have modified the system of Shelton et al (termed SNAP) of neurological evaluation after TBI in mice (J Neurosci Methods, 168, 431-442, 2008) for use in our studies with rats. The system consists of 8 different tests that are primarily observational categories. We construct our score by using 6 out of the eight categories: Interactions (or avoidance to being handled upon removal from the cage), Cage Grasp (the manner in which the animal releases from holding onto the cage bars on the top of the cage), Visual Placing (the manner in which the animal reaches for an approaching table top), Gait and Posture (noting abnormalities while the animal is moving freely about a space), presence or absence of Head Tilt , and Baton (level of coordination used to grasp a stick with all four feet). Each category is scored on a scale of 0 to 5 and added to produce a neuroscore. For the baton, which is the proper size and weight for the animal to grasp comfortably, we use a wooden dowel (currently ¼ inch diameter x 3 ft long). With the exception of being allowed to move about freely on a counter top briefly and grasping a baton, the above tests or categories of observation all occur in situations commonly experienced by the animals during normal husbandry practices.

***Neuroscore results following CCI:*** <u>The Neuroscore</u> is about 0.75 units better in xenon-treated-TBI animals than air (control)-TBI animals on both day 2 or day 3. However, the standard deviation (SD) is ~

2 on day 2, so one would need about 100 animals per experimental group to achieve statistical significance. Day 3 has an average SD of ~ 1, which is within the design parameters of the original N of 14. The requirement to over-select this one approach to find anything positive (i.e., the Bonferroni correction) suggests it is unlikely to hold, and thus provides only weak justification moving forward with experiments using this model. Assessment of total errors also suggests too little signal/noise.

*Table 1: Neuroscore on Days 2 and 3 post-TBI/sham with or without xenon treatment.* . SX, sham treated with xenon; SA, sham treated with air; TX = trauma treated with xenon; TA = Trauma treated with air. N= 4/group.

| **Neuroscore** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 2 | Day 3 | Total | Avr Day 2 | Avr Day 3 | Avr D2+D3 | SD Day 2 | SD Day 3 | SD Total |
| SX | 0 | 0 | 0 | 0.0 | 0.3 | 0.3 | 0.0 | 0.5 | 0.5 |
| SX | 0 | 0 | 0 | | | | | | |
| SX | 0 | 0 | 0 | | | | | | |
| SX | 0 | 1 | 1 | | | | | | |
| SA | 0 | 0 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| SA | 0 | 0 | 0 | | | | | | |
| SA | 0 | 0 | 0 | | | | | | |
| SA | 0 | 0 | 0 | | | | | | |
| TX | 1 | 2 | 3 | 2.8 | 2.3 | 5.0 | 1.3 | 1.3 | 2.2 |
| TX | 3 | 1 | 4 | | | | | | |
| TX | 3 | 2 | 5 | | | | | | |
| TX | 4 | 4 | 8 | | | | | | |
| TA | 1 | 3 | 4 | 3.5 | 3.0 | 6.5 | 2.4 | 0.8 | 2.9 |
| TA | 2 | 2 | 4 | | | | | | |
| TA | 5 | 4 | 9 | | | | | | |
| TA | 6 | 3 | 9 | | | | | | |
| | | | | | | | | | |
| | | | delta X | 0.75 | 0.75 | 1.50 | | | |
| | | | Aver SD | 1.82 | 1.04 | 2.52 | | | |

**2c: Rotarod assessment:** Rotarod testing is implemented using the programmed, accelerating Rotarod. The duration in seconds at the point at which the animal either completes the task (maximum of 2 minutes), falls from the rods, or grips the rods and spins for two consecutive revolutions rather than actively walks, is recorded as the Rotarod score. Post-injury assessment begins at 24 hours post-injury and is performed every 24 hours thereafter to complete 3 days total. The exact testing schedule can change based on our experience with these animals.

***Results with Rotarod following CCI:*** The most stable measure seems to be the inability to complete at least one of the three runs (i.e., reach 2 min) in each of the seven days. This measure has an $r^2$>0.9 with the medians of the best time on the rod/day, and has the advantage of being conceptually simpler. By this measure, the failure rates are (T=TBI; S=Sham, A=air, X=xenon): TX 7,5,5,2; TA 7,6,3,1, SX, 0,0,0,0; SA, 6,3,0,0. The worst TA animal actually failed training as well, and should likely be excluded for cause, thus amplifying the advantage of the air-treated animals over the Xe treated ones. In addition to this type of problem reducing power, other concerns with this assay have arisen during this study: (i) 2/8 of the shams at least occasionally fail, indeed one fails more than 5/8 of the TBI animals; (ii) several animals (injured and uninjured) appeared to our veterinarian as being either very uncomfortable/vocal on the Rotorod, while others appeared not to care if they fell, e.g., turning around, jumping onto a

narrow ledge, walking backwards, lowering themselves, etc. Thus, this assay seems less appropriate for these animals, possibly due to their improved vision relative to other rats. We will continue to evaluate this test in the initial fluid percussion experiments, and may discontinue it if these issues persist.

Given the noise inherent in the system, we do not believe it would be possible to reach significance in an N of 14 assuming draws from an equal population (i.e., the effect would have to be so large in the remaining animals that it is highly unlikely we would have gotten the data above from the first four).

**2d. Beam walk test.** The elevated beam is constructed so as to detect non-compensatory foot-fault deficits in brain-injured rats (Dragonfly, Inc., Ridgely, WV). The length of the beam is tapered such that the starting width of the walking surface is 5.5 cm and the ending width is 1.5 cm. Along each side is a 2 cm wide ledge positioned 2 cm below the beam surface. The ledge allows the rat to avoid compensatory changes in posturing and weight distribution. Each ledge is equipped with mechanical sensors and digital recorders to detect the number of left or right steps onto it. Each step onto the ledge is recorded as a foot-fault. There is a platform at the starting end and a darkened box at the finishing end. Once in the darkened box, the animal will be allowed to stay there for 30 seconds, for positive reinforcement. We monitor beginning at 24 hours post-injury and every 24 hours thereafter to complete 3 days total. The exact testing schedule can change based on our experience with these animals. Foot-faults are added from three consecutive runs.

***Results on Beamwalk following CCI***: On Beamwalk, animals are tested for their ability to traverse a tapering beam, while footfaults are counted automatically on contact with a surrounding ledge. Overall, xenon treated animals do worse than controls the first few days (Figure 1), then catch up and precede at the same pace. Given this data and the noise inherent in the system, we do not believe it would be possible to reach significance in an N of 14 assuming draws from an equal population (i.e., the effect would have to be so large in the remaining animals that it is highly unlikely we would have gotten the data above).

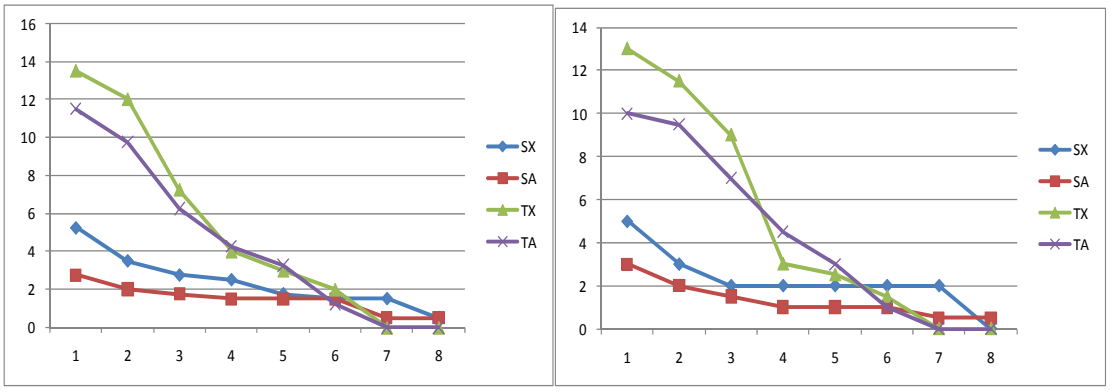


**Figure 1: Xenon does not reduce errors in the Beamwalk:** *Left panel*: Average cumulative remaining errors. *Right panel:* Median cumulative remaining errors. Y axis = errors; X axis = days, Day 1 = day of surgery. SX, sham treated with xenon; SA, sham treated with air; TX = trauma treated with xenon; TA = Trauma treated with air. N= 4/group.

**2e: Cylinder reach test.** Animals with unilateral injuries reach preferentially with the paw on the non-injured side. Rats are placed in a clear cylinder and allowed to explore for 5 minutes. Wall touches and duration of contact with each front paw are measured. Test is conducted on days 1 and 3 post injury.

***Results of cylinder test following CCI:*** The cylinder test was filmed but has not yet been scored.

**Task 3. Determine the effect of inhaled xenon on behavior and histopathology in long-term fluid percussion (FP) and controlled cortical impact (CCI) rat models of TBI compared to controls.**
We are proceeding with the most efficient approach to both short- and long-term outcome measures. Our adaptive approach informs the approach to undertaking long-term studies. If we the FP short-term study is positive, we will proceed with the FP long-term study. If it is negative, or if it is neutral as is the CCI study, we can reconsider either model or a different model at that time. We note, however, that the substantial CCI-induced hippocampal injury appears unaffected at best in Xenon-treated animals, suggesting that Xenon is unlikely to show substantial protection in long-term studies in the CCI model. Thus, in neither case does running the CCI long-term study seem prudent.

**KEY RESEARCH ACCOMPLISHMENTS:**

- Designed and manufactured a unique xenon-recirculation box in which the concentration of xenon and oxygen are reproducibly and accurately controlled.
- Conducted the first in vivo experiments to assess whether xenon provides neuroprotection following TBI in a rat model.

**REPORTABLE OUTCOMES**:

- Designed (primarily by colleague Dr. Jose Venegas) and manufactured a unique xenon-recirculation box in which the concentration of xenon and oxygen are reproducibly and accurately controlled. This can be used for a variety of xenon-related experiments.

**CONCLUSION:** We have successfully established a reliable and reproducible model for xenon administration in rat models of TBI, and conducted these first *in vivo* experiments. We have also established procedures for different models of TBI and their assessment through histopathologic and behavioral outcomes. To date our data suggest that the CCI model induces substantial injury, particularly to the hippocampus, making this model less appropriate to study TBI for neuroprotection. The histopathologic and behavioral data suggest that there was no significant improvement in short-term histopathologic and some behavioral outcomes, and perhaps xenon-treated animals fared worse for these outcomes early after injury. We are undertaking the FP model for these studies. The type and extent of injury may be as important as any therapeutic agent or the timing and duration of its administration. The outcome measures in rats can be problematic and may be of questionable relevance in humans. However, such work is necessary to identify conditions and models with which to work. While it is too early to say definitively, it is possible that xenon may not be effective in severe TBI or that it could work synergistically with other agents.

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**APPENDICES:** n/a